Protein-chemical analysis of pertussis toxin reveals homology between the subunits S_2 and S_3, between S_1 and the A chains of enterotoxins of *Vibrio cholerae* and *Escherichia coli* and identifies S_2 as the haptoglobin-binding subunit

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The purified toxin of *Bordetella pertussis* was dissociated in 5 M urea in the presence of immobilized haptoglobin. The toxin was dissociated in free S_1, free S_5 and the free complexes S_2-S_4 and S_3-S_4, with S_2-S_4 as the only haptoglobin-binding moiety, identifying S_2 as the haptoglobin-binding protein. Partial NH_2-terminal amino acid sequences were obtained from the dissimilar toxin subunits, after separation by SDS-polyacrylamide gel electrophoresis followed by electroblotting onto polybrene-coated glass-fiber sheets. The sequences reveal extensive homology of the N-terminal portions of the constitutive subunits S_2 and S_3 and between S_1 and the enterotoxin A chains of *Vibrio cholerae* and *Escherichia coli*.

Pertussis toxin *Cholera toxin* (E. coli) *Haptoglobin binding* *Protein electroblotting* *Protein sequencing*

1. INTRODUCTION

Pertussis toxin is a protein toxin produced by phase I, virulent strains of *Bordetella pertussis* [1–3]. It can be isolated and purified from the culture medium of the bacteria [4–7] and it was shown to induce a whole range of biological activities including leukocytosis and lymphocytosis [8,9], histamine sensitization [10], potentiation of insulin secretion [11], lethality, mitogenicity and adjuvant effects [12].

PT is an oligomeric protein consisting of six subunits, five of which are different from each other in molecular mass by polyacrylamide gel analysis in the presence of SDS. Treating the PT with increasing concentrations of urea causes the toxin to gradually dissociate into its components and has allowed a partial reconstruction of the interaction between the different subunits [13]. Thus it has been proposed that PT forms an A-B structural organization similar to that of other bacterial toxins: the A (active) protomer being subunit S_1 (M_r 28000) and catalyzing the ADP-ribosylation of the inhibitory guanyl nucleotide component of the adenylate cyclase system (G-protein) [14–16]; the B (binding) oligomer consisting of five subunits: one S_2 (M_r 23000), one S_3 (M_r 22000), two S_4 (M_r 11700) and one S_5 (M_r 9300) and promoting the binding of the native toxin to receptor molecules allowing the A protomer to reach its site of action within the cell.

As a further study of the molecular nature of the toxin subunits and the interactions between them, we have analyzed the subunit interactions in the

Abbreviations: PT, pertussis toxin; DEAE, diethylaminoethyl; HPLC, high-performance liquid chromatography

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presence of urea and immobilized haptoglobin. We also provide the NH_2-terminal sequence of the different subunits and compare the results both with each other and with the protein sequences stored in the protein sequence data banks. Points of homology are discussed.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Chemicals and solvents used in this study were either analytical grade or HPLC grade. Urea was from Merck, FRG. DEAE-Sepharose CL-6B was from Pharmacia, Sweden. Haptoglobin was purchased from Calbiochem-Behring, USA. It was immobilized onto Sepharose according to the procedure of Irons and MacLennan [17]. Polybrene was obtained from Janssen Biochimica, Belgium and used without further purification. Glass-fiber sheets (GF/C) were from Whatman, England. They were cut from 57 × 46 cm sheets to the size of the gel.

The protein reference mixture was the Bio-Rad molecular mass standard mixture. Fluorescamine was a product from Fluka, Switzerland. Solvents and reagents used for the gas-phase sequenator were purchased from Applied Biosystems, USA. Solvents used for phenylthiohydantoin-amino acid analysis were from Carlo Erba, Italy.

2.2. *Toxin preparations*

One preparation of pertussis toxin was a commercially available toxin from List Biological Laboratories, USA. A second toxin preparation was isolated from the Tohama strain, phase I. Therefore *Bordetella pertussis* bacteria were grown for 50 h at 36°C in modified Stainer-Scholte medium containing heptakis (2,6-*O*-dimethyl) β-cyclodextrin [18,19]. Cells were harvested by centrifugation and the toxin was purified from the culture supernatant according to the procedure of Sato et al. [6]. The purity of the PT was checked by electrophoresis in 7.5% polyacrylamide gels at pH 3.8 [20]; gels were fixed and stained for 16 h at room temperature with 0.01% Coomassie blue G 250 in 12.5% trichloroacetic acid.

2.3. *Dissociation and separation of the PT subunit*

PT was exposed to 5 M urea for 6 h at 4°C and applied to a column of haptoglobin-Sepharose 4B in 0.017 M phosphate buffer (pH 8.3) containing 5 M urea. The protein fraction which was eluted with the same buffer was chromatographed on a DEAE-Sepharose CL-6B column in the same urea solution. The material retained by the affinity adsorbent was eluted by a stepwise change of the buffer to 0.5 M NaCl/0.1 M Tris-HCl (pH 10.0) containing 3 M KSCN. The subunits of PT were analyzed on SDS - containing 15% polyacrylamide gels as described by Laemmli [21]. The gels were stained with silver according to Wray et al. [22].

2.4. *Electroblotting on polybrene-coated glass-fiber sheets and amino acid sequence analysis*

25 μg of the purified PT were separated on a 15% gel and the separation was terminated when the bromophenol blue reached the edge of the gel. The gel was mounted immediately into the blotting sandwich, containing two layers of polybrene-coated glass-fiber sheets (Whatman GF/C) placed back to back on the anodic side of the gel. The electrotransfer was carried out as described by Vandekerckhove et al. [23]. Blotting was stopped after 24 h, the glass-fiber sheets were washed three times in 25 mM NaCl, 10 mM borate buffer, pH 8.0, and dried. Immobilized proteins were detected on both sheets using a dilute fluorescamine stain (1 mg/600 ml acetone). Results were recorded by photography under UV light using a Polaroid camera and a mineral light lamp UV SL 20 (San Gabriel, CA). The areas of the glass-fiber carrying the proteins were cut out from both sheets and placed into the reaction chamber of the gas-phase sequenator (Applied Biosystems, USA). The sequenator was run essentially as described by Hewick et al. [24]. The stepwise liberated phenylthiohydantoin-amino acids were identified by HPLC analysis using the column and gradient system described by Hunkapiller and Hood [25].

3. RESULTS AND DISCUSSION

We have taken advantage of the haptoglobin-binding property of PT [17] to develop a rapid purification procedure for the five subunits. PT (Tohama strain) was treated for 6 h at 4°C in urea buffer (see section 2) and loaded onto a column of haptoglobin-Sepharose. The protein fraction which was not retained in these conditions, was analyzed by SDS-polyacrylamide gel elec-

trophoresis and revealed the presence of subunits S_1, S_3, S_4 and S_5 (fig.1)

The material binding to haptoglobin was eluted by adding 3 M KSCN to the column buffer. This fraction contained equal amounts of subunits S_2 and S_4 (fig.1). Proteins from the non-adsorbing fraction were further purified on DEAE-Sepharose CL-6B using the 5 M urea buffer (see above). Isocratic elution of the DEAE column with this buffer yielded three components (peak I, II and III). SDS gel analysis of these fractions showed that peak I contained a mixture of S_3 and S_4 equally present, while peaks II and III contained subunits S_1 and S_5, respectively (fig.2). Each of the dimers, S_2-S_4, obtained as the haptoglobin-binding fraction, and S_3-S_4, obtained as the non-adsorbing peak I, could be dissociated in 8 M urea and separated into their constituent monomers as

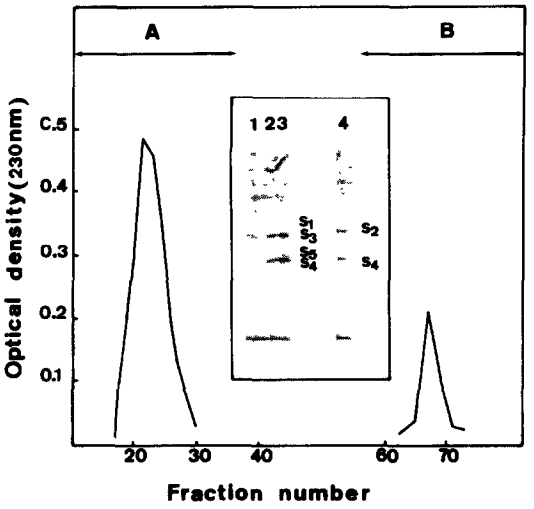

Fig.1. Haptoglobin-Sepharose 4B affinity chromatography of partially dissociated PT. A solution of PT (0.5 mg/0.5 ml) in 0.1 M sodium phosphate, pH 7.0, 0.5 M NaCl, 2 M urea, was dialyzed during 6 h at 4°C against 0.017 M phosphate buffer (pH 8.3) containing 5 M urea. The dissociated toxin was loaded on a haptoglobin-Sepharose 4B column (1 × 2.5 cm) which was equilibrated with the dialysis buffer. (A) Peak fractions obtained by eluting the column with the equilibration buffer; (B) material eluting with a step gradient of the first buffer to which NaCl (0.5 M) and KSCN (3 M) was added and adjusted to pH 10.0. Inset, Separation pattern of the affinity separated subunits after SDS-polyacrylamide gel electrophoresis. Lanes: 1, fraction 20; 2, fraction 25; 3, fraction 27; 4, fraction 67.

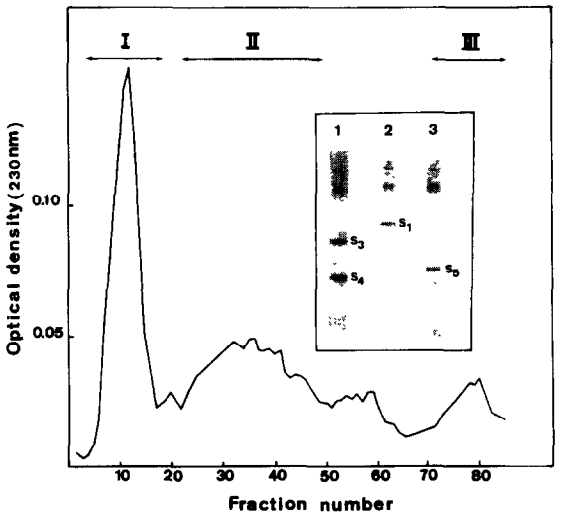

Fig.2. DEAE-Sepharose CL-6B chromatography of the non-haptoglobin binding fraction. Fractions of peak A (fig.1) were combined and loaded on a DEAE-Sepharose CL-6B column (1 × 3 cm) equilibrated in 0.017 M phosphate buffer (pH 8.3) containing 5 M urea. The column was isocratically eluted with a flow rate of 15 ml/h. Fractions were combined as indicated (peaks I–III), dialyzed against 0.05 M ammonium bicarbonate and lyophilized. Aliquots were removed and analyzed by SDS-polyacrylamide gel analysis (inset). Lanes: 1, proteins of peak I; 2, proteins of peak II; 3, proteins of peak III.

described by Tamura et al. [13] using DEAE ion-exchange chromatography.

These results differ from those reported previously by Tamura et al. [13] where only S_1 was released from the PT complex (protomer A) leaving the B oligomer (S_2-S_4, S_3-S_4 and S_5) as the intact haptoglobin-binding complex. In contrast, we find that under identical dissociating conditions as described before, PT dissociates into free S_1, free S_5 and the two free dimers S_2-S_4 and S_3-S_4 with only S_2-S_4 being retained by the haptoglobin column. These results suggest that in 5 M urea S_2 is the only haptoglobin-binding subunit. It is also possible that complex formation between S_2 and S_4 is necessary to provide the haptoglobin-binding capacity to S_2. Further protein chemical characterization of the PT subunits was made by partial amino acid analysis. We therefore subjected the urea purified proteins to automated amino acid sequence analysis using a gas-phase sequenator. We obtained extremely poor sequence

information due to very low initial coupling yields (less than 1%). This is most probably due to extensive carbamylation taking place during long exposure to high concentrations of urea.

The problem of NH_2-terminal blocking was circumvented by making use of the recently developed technique of protein electroblotting on polybrene glass-fiber sheets after separation of the proteins on SDS-polyacrylamide gels [23]. This technique appeared to be extremely valuable in this particular case since we could now easily separate small amounts of the expensive toxin preparations.

25 μg of the toxin were separated on a 15% SDS-polyacrylamide gel and electroblotted onto glass-fiber sheets. The fluorescamine-stained pattern of the immobilized proteins is shown in fig.3. A parallel experiment with equal amounts of protein allowed one to measure the amount of immobilized protein (between 80–150 pmol) and to

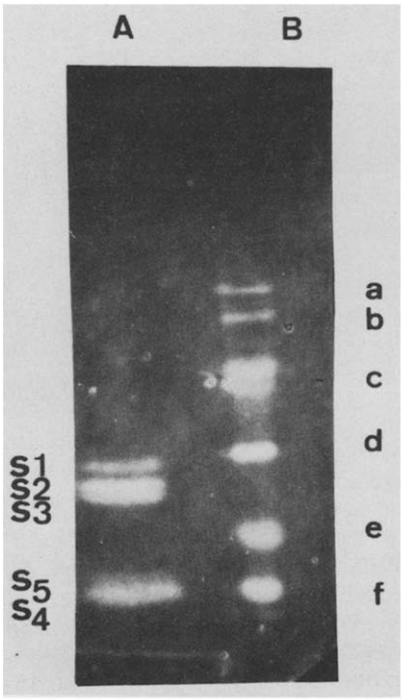

Fig.3. Electroblot of the PT subunits from SDS-polyacrylamide gel onto polybrene-coated glass-fiber. Lanes: A, the PT subunits; B, a standard molecular mass protein mixture containing phosphorylase *b* (a), bovine serum albumin (b), ovalbumin (c), carbonic anhydrase (d), soybean trypsin inhibitor (e) and lysozyme (f). Proteins were separated on a 15% polyacrylamide gel.

calculate sequencing efficiencies. Each of the bands was sequenced for 25 steps (S_2 only for 15 steps) and amino acid sequences could unambiguously be allocated to each of these subunits as shown in fig.4. This was generally possible for approx. 20 residues (± 10% of the total sequence). For subunit S_2, we obtained less sequence information due to low initial coupling yields in the sequence run.

The blotting experiment was also repeated with commercially available toxin (List Biological Laboratories, USA; strain unknown). Both strains reveal identical NH_2-terminal sequences. Subunits S_2 and S_3 are very homologous in their NH_2-terminal sequences and even identical in positions 3–8. This homology is in line with the very similar amino acid composition reported previously for both subunits [13] and with their ability to form an equally stable complex with the same subunit S_4. Since S_2-S_4 and S_3-S_4 differ in their haptoglobin-binding capacity (see above), the haptoglobin-binding site of S_2 should be located in the sequence where S_2 and S_3 differ from each other. The present studies only cover the first ten amino acid residues of S_2 and further experiments will be necessary to carry out a more extended homology analysis. No further significant homology was found between the NH_2-termini of the remaining subunits. The NH_2-terminal sequence of subunit S_4 was found to be in complete agreement with the sequence which was published recently [26].

We have also carried out a comparative study with the protein sequences stored in the Protein Sequence Data Bank (release 6) of National Biomedical Research Foundation (NBRF), Washington, DC, USA, using the FASTP pro-

S_1: 1 D D P P A T V Y R Y D S R P P E D V F Q N G F

S_2: 1 S T P G I V I P X Q E Q I

S_3: 1 V A P G I V I P P K A L F T Q Q G G A

S_4: 1 D V P Y V L V K T N M V V T X V

S_5: 1 I Y S P A D V A G L P T H L Y K N F T V Q

Fig.4. NH_2-terminal sequences of the different subunits of pertussis toxin. X denotes a position where no amino acid could be detected.

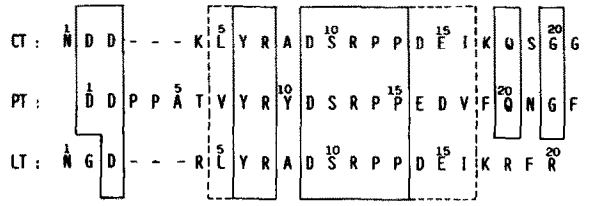

Fig.5. Comparison of the NH_2-terminal sequence of subunit S_1 of pertussis toxin with the enterotoxin A chains of *V. cholerae* (CT) and *E. coli* (LT). Amino acids are arranged as to obtain the highest degree of homology. Identical amino acids are shown in boxes. Conserved amino acid exchanges between the proteins are given in dotted lines.

gram. Of the five NH_2-terminal sequences compared, S_1 showed a high degree of homology with the A subunit of *V. cholerae* toxin (52.7% identity in a 17 amino acid overlap) and to a lesser extent with the A chain of the *E. coli* heat-labile enterotoxin (58.3% identity in a 12 amino acid overlap) (fig.5). These results are in agreement with the known functional homology between PT subunit S_1 and the A protomer of the other toxins, both identified as catalyzing the ADP-ribosylation of G-protein and producing a steady-state level of activation of the adenylate cyclase [14–16].

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